Neoplastic Transformation of Cultured Mammalian Cells by Estrogens and Estrogenlike Chemicals

Takeki Tsutsui[1] and J. Carl Barrett[2]

[1]Department of Pharmacology, School of Dentistry at Tokyo, The Nippon Dental University, Tokyo, Japan; [2]Environmental Carcinogenesis Program, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina

Estrogens are clearly carcinogenic in humans and rodents but the mechanisms by which these hormones induce cancer are only partially understood. Stimulation of cell proliferation and gene expression by binding to the estrogen receptor is one important mechanism in hormonal carcinogenesis; however, estrogenicity is not sufficient to explain the carcinogenic activity of all estrogens because some estrogens are not carcinogenic. Estrogens are nonmutagenic in many assays but exhibit specific types of genotoxic activity under certain conditions. We have studied extensively the mechanisms by which estrogens induce neoplastic transformation in a model *in vitro* system and our findings are summarized in this review. 17β-Estradiol (E_2) and diethylstilbestrol (DES) and their metabolites induce morphological and neoplastic transformation of Syrian hamster embryo (SHE) cells that express no measurable levels of estrogen receptor. Treatment of the cells with E_2 or DES fails to induce DNA damage, chromosome aberrations and gene mutations in SHE cells but results in numerical chromosome aberrations (aneuploidy) that could arise from microtubule disruption or disfunction of mitotic apparatus. Estrogen-induced genotoxicity is detected in cells following treatment with estrogen metabolites or following exogenous metabolic activation of estrogens. The estrogens induce DNA adduct formation that is detected by ^{32}P-postlabeling. Both aneuploidy induction and DNA damage caused by DNA adduct formation correlate with the estrogen-induced cell transformation and may be important in hormonal carcinogenesis. We propose that multiple effects of estrogens acting together cause genetic alterations leading to cell transformation. — *Environ Health Perspect* 105(Suppl 3):619–624 (1997)

Key words: estrogens, estrogen metabolites, cell transformation, genotoxicity

Estrogen use has been associated with an increased risk for breast cancer and endometrial cancers in women (*1–4*). Various natural and synthetic estrogens also induce mammary, pituitary, cervical, uterine, and renal tumors in rodents (*4–5*). Diethylstilbestrol (DES), a synthetic estrogen, is well known to be carcinogenic to humans (*6*). The cellular and molecular mechanism(s) whereby estrogen-induced neoplastic events occur have not been fully elucidated, but there is strong evidence that estrogens are epigenetic carcinogens, acting via a promoting effect related to cellular proliferation, mediated through the estrogen receptor (*7–15*). However, it has been shown that estrogenic activity is not sufficient to explain the carcinogenic activity *in vivo* and *in vitro* under certain experimental conditions. Another mechanism, related to mutagenic changes, has been suggested in studies of estrogen-induced carcinogenesis (*16–24*).

The application of cell cultures to study carcinogenic mechanisms of chemical/physical carcinogens can provide insights into the cellular and molecular mechanisms of carcinogenesis, which is difficult in whole-animal or human systems. We have used Syrian hamster embryo (SHE) fibroblast cell cultures as a model system to study the ability of estrogens to directly transform cells.

Morphological and Neoplastic Transformation *in Vitro* by Estrogens and Estrogenlike Chemicals

Morphological and neoplastic transformation of SHE cells is induced by DES, 17β-estradiol (E_2) and other estrogens. We observed that DES and E_2 induce transformation of hamster cells that is indistinguishable from that induced by other chemical carcinogens such as benzo[*a*]pyrene (*19,25*). Sarcomas are also induced in Syrian hamsters *in vivo* following subcutaneous injection of DES (*26*). SHE cells do not express measurable levels of estrogen receptor and estrogen treatment is not mitogenic to the cells (*27*). Thus, estrogenic activity of the compounds can be excluded in this *in vitro* assay. The cells do, however, have the ability to metabolize estrogens (*28,29*), and the role of metabolic activation in the carcinogenesis activity of estrogens in this model is under investigation as discussed later.

The role of mutagenesis in the neoplastic transformation of SHE cells by estrogens has been studied extensively. We have demonstrated that treatment of SHE cells with DES or E_2 induces cell transformation without measurable gene mutations, unscheduled DNA synthesis (UDS) or structural chromosome aberrations (*19,20,23*). Under the same conditions, both estrogens induce a specific type of genetic change, i.e., aneuploidy. Chromosome losses and gains are induced (*20,23*), suggesting a nondisjunctional mechanism involved in the transforming activity. Structural analogues of DES have also been tested in this cell transformation system (*25,30*). Like DES, tetrafluorodiethylstilbestrol (TF-DES) and dimethylstilbestrol (DMS) induce morphological transformation of SHE cells. The transformation frequency of DMS is much less than that of DES and TF-DES. Hexestrol (HEX) and dimethoxydiethylstilbestrol (DM-DES) do not transform these cells. There is a good association between the metabolic conversion of DES analogues via a peroxidase-mediated

This paper was presented in part at the Workshop on Hormones, Hormone Metabolism, Environment, and Breast Cancer held 28–29 September 1995 in New Orleans, Louisiana. Manuscript received at *EHP* 6 June 1996; manuscript accepted 13 September 1996.

Address correspondence to Dr. J.C. Barrett, National Institute of Environmental Health Sciences, PO Box 12233, Research Triangle Park, NC 27709. Telephone: (919) 541-2992. Fax: (919) 541-7784. E-mail: barrett@niehs.nih.gov

Abbreviations used: DES, diethylstilbestrol; α-dienestrol, *trans,trans*-dienestrol; β-dienestrol, *cis,cis*-dienestrol; DMS, dimethylstilbestrol; DM-DES, dimethoxydiethylstilbestrol; E_1, estrone; E_2, 17β-estradiol; E_3, estriol; HEX, hexestrol; MN, micrococlei; 8-OH-dG, 8-hydroxy-2′-deoxyguanosine; 16α-OH E_1, 16α-hydroxyestrone; 2-OH E_1, 2-hydroxyestrone; 2-OH E_2, 2-hydroxyestradiol; 4-OH E_2, 4-hydroxyestradiol; PMS, postmitochondrial supernatant; SHE, Syrian hamster embryo; TF-DES, tetrafluorodiethylstilbestrol; UDS, unscheduled DNA synthesis.

oxidative pathway and their ability to induce cell transformation. DES, DMS, and TF-DES can all be metabolized by peroxidase. In contrast, HEX and DM-DES are not metabolized via this pathway (*25*), suggesting that DES metabolism is important in its carcinogenicity. The peroxidative-mediated metabolism of DES that operates in SHE cells is also the major pathway of DES metabolism in the known DES target tissue [e.g., adult (*31*) or fetal uterus (*32*)] (*25*).

Treatment of SHE cells with DES in the presence of exogenous metabolic activation with rat liver postmitochondrial supernatant (PMS) enhances morphological transformation in a dose-dependent manner (*33*). Exposure of SHE cells to DES under the same conditions with exogenous metabolic activation induces DNA damage (determined by UDS) (*21*), and somatic mutation at the Na^+/K^+-ATPase locus (*33*). SHE cells peroxidatively metabolize DES to *cis,cis*-dienestrol (β-dienestrol) (*29*), which does not induce UDS by itself (*21*). More β-dienestrol is formed in cells treated in the presence of rat liver PMS.

β-Dienestrol can potentially form a phenoxyradical intermediate via a peroxidase-mediated pathway, and this intermediate may bind to DNA and induce UDS.

DES exhibits positive activity in other cell transformation systems as well. Fitzgerald et al. (*34*) demonstrated in the BALB/c 3T3 cell transformation system that DES displays transforming activity with no measurable induction of gene mutation at the Na^+/K^+-ATPase locus. Transformation frequency of the cells is enhanced by DES when treated with DES in the presence of rat hepatocytes that are freshly prepared. Rinehart et al. (*35*) showed that chronic exposure to DES of human endometrial stromal cells with a temperature-sensitive SV40 large T antigen induces a dose-dependent increase in the immortalization of cells, which is determined by the ability to grow at the restrictive temperature. Moreover, the increase in cell proliferation at the restrictive temperature is concurrent with alterations in *p53* in the cells. As immortalization is an important step in the carcinogenesis process, and immortalization of human cells may be analogous to initiation of rodent cells, DES could act as an initiator in the carcinogenic process of human cells (*35*).

We have studied the ability of estradiol (E_2) and different metabolites of E_2 to induce cell transformation. E_2 metabolism is similar in rats and in humans. E_2 is initially oxidized to estrone (E_1), followed by hydroxylations at positions C-2 and 16α that are mutually exclusive (*5*). The principal products of 16α-hydroxylation are 16α-hydroxyestrone (16α-OH E_1) and estriol (E_3). 2-Hydroxylation yields the catechol estrogen 2-hydroxyestrone (2-OH E_1) (*36*). P450 multisubstrate monooxygenases catalyze the oxidative metabolism of estrogens, and estrogen 2- and 16α-hydroxylases play a major role in the metabolism of these estrogens (*37*). E_2 is also converted to catechol estrogen, postulated to arise from E_2 via the 4-hydroxylation pathway, which is similar to the 2-hydroxylation pathway (*38*). The catechol estrogens e.g., 2-OH E_1, 2-hydroxyestradiol (2-OH E_2), and 4-hydroxyestradiol (4-OH E_2) seem to be further oxidized to quinones that covalently bind to DNA (*39*).

We examined the transforming activity of these estrogens using the SHE cell assay system. Treatment of SHE cells with E_1, E_2, 16α-OH E_1, 2-OH E_1, or 2-OH E_2 induces morphological transformation of cells in a dose-related manner. Exposure to E_3 fails to elicit SHE cell transformation (unpublished data). Higher transforming activity is observed in cells treated with 16α-OH E_1 or 2-OH E_1, when compared to other estrogens. 16α-OH E_1 could be capable of inducing UDS and anchorage-independent growth in mouse mammary epithelial cells (*40*). Additionally, 16α-OH E_1 binds covalently not only to nucleohistones *in vitro* (*41*), but also to nuclear regulatory proteins, specifically the estrogen receptor, in estrogen target cells (*42*). This may disturb normal gene functions, possibly participating in the transformation process (*41*). Elevated levels of 16α-hydroxylation are detected in breast tissue from women with breast cancer (*43*) and in mouse strains with a high incidence of mammary tumor formation (*44*). However, the high frequency of morphological transformation of cells induced by 2-OH E_1 could be due to genotoxicity of 2-OH E_1 or its metabolites converted in SHE cells, because treatment with 2-OH E_1 induces chromosome aberrations in SHE cells (unpublished data). E_2 induces morphological transformation of BALB/c 3T3 cells, a mouse fibroblast cell line having 2- and 4-hydroxylase activity. The transformation efficiency does not increase with increasing hormonal potency of the estrogens examined, but correlates well with the relative rates of catechol estrogen formation (*45*).

Tamoxifen, toremifene, and ICI 164,384 are positive in the SHE cell transformation assay as well (*46*). Tamoxifen, a triphenylethylene nonsteroidal antiestrogen, is a structural analogue of DES and exerts mixed or partial agonist/antagonist effects with estrogens. Toremifene is a new triphenylethylene nonsteroidal antiestrogen; its molecular structure closely resembles that of tamoxifen. Toremifene differs from tamoxifen by the presence of a chlorine atom at the end of the ethyl side chain. ICI 164,384 is the 7α-alkylamide analogue of E_2, and a new steroidal antiestrogen with complete pure antagonistic properties (*47*). The results confirm that hormonal effects are not implicated in cell transformation. Rather, a role for estrogen metabolism seems to be important in estrogen-induced cell transformation or carcinogenesis. No reports on the cell transforming activity and carcinogenicity of other estrogen blockers, e.g., EM 800 and ICI 182,780, are available.

Mechanisms of Cell Transformation by Estrogens

Aneuploidy Induction

DES induces numerical chromosome changes (aneuploidy) in SHE cells (*20*). The aneuploidy induction occurs at nontoxic doses and correlates with the ability to induce cell transformation with parallel dose–response curves. Treatment of synchronized cultures with DES results in a cell cycle-dependent induction of aneuploid cells that parallels the induction of cell transformation, with the greatest level observed following treatment during mitosis. Parallel dose–response curves for cell transformation and aneuploidy induction by DES are observed when the synchronized cultures are treated during the mitotic phase of the cell cycle (*20*). A nonrandom chromosome gain accompanies DES-induced immortalization and tumorigenic conversion of SHE cells (*48*). These suggest that DES-induced aneuploidy is mechanistically involved in estrogen-induced cell transformation and possibly in carcinogenesis (*7,22*). E_2 also induces a dose-dependent increase in the frequencies of aneuploid cells, corresponding to the inducibility of morphological transformation (*23*).

E_2 and DES bind and disrupt polymerization of microtubules in cultured mammalian cells (*49–53*). DES inhibits *in vitro* assembly of microtubules purified from porcine brain (*54*), and induces a decrease in the number of spindles and cytoplasmic microtubule fibers in SHE cells (*49*) and Chinese hamster V79 cells (*50*). E_2 has no ability to interact with microtubules or

microtubule protein *in vitro* (*54*), but the quinone metabolites of both E_2 and DES bind covalently to the C-terminal regions of β-tubulin, which are important in regulation of microtubule assembly and disruption (*55*). E_2 exhibits microtubule-disrupting activity both in estrogen receptor-positive and receptor-negative human breast cancer cell lines (*51*). The disrupting activity is demonstrated also in V79 cells (*51,52*), which have little capability of metabolizing xenobiotics (*56*). These findings suggest that E_2 itself induces microtubule disruption independent of its binding to estrogen receptor. Therefore, E_2-induced microtubule disruption in living cells seems to be due to a more complex involvement with factors regulating microtubule assembly, such as Ca^{2+}, microtubule accessory proteins, the calcium regulatory protein calmodulin, adenylate cyclase, or the protein kinases activated by the cyclic nucleotides (*57–59*). Microtubule-disrupting activities of E_2 and its metabolites in living cells vary with their chemical structures. Aizu-Yokota et al. (*53*) have examined the activity in V79 cells by the indirect immunofluorescence method using anti-β-tubulin antibody and determined the rank-order of the potencies as follows: $E_2 \approx$ 2-OH $E_2 >$ 4-OH $E_2 >$ 16α-OH $E_1 \approx$ 2-OH $E_1 > E_1 \approx E_3$. 16α-OH E_1 and 2-OH E_1 exhibit activities about one-fourth to one-fifth that of E_2. The disruptive activities of E_1 and E_3 are negligible when compared to that of E_2. Functional or conformational change in microtubule organization could lead to chromosomal nondisjunction, aneuploidy induction, and cell transformation.

Tamoxifen, toremifene, and ICI 164,384 induce aneuploidy in SHE cells with no increases in the frequency of chromosome aberrations (*46*). Tamoxifen binds to calmodulin and acts as a calmodulin antagonist (*60*). Sargent et al. (*61*) reported that both unipolar spindles and incompletely elongated spindles were observed in cultured hepatocytes from rats treated with tamoxifen, as well as in calmodulin-defective mutants in yeast (*62*). Some calmodulin is associated with the spindle pole body and plays an important part in the proper function of mitotic spindles (*62*). Tamoxifen-induced aneuploidy may be due to the inhibitory effect of calmodulin by tamoxifen.

Formation of Micronuclei

Both DES and E_2 induce the formation of micronuclei (MN) in cultured mammalian cells (*63–67*). MN enclose acentric chromosome fragments or whole chromosomes that do not become incorporated into the main nuclei after cell division. MN are believed to arise from acentric chromosomal fragments or from chromosomes lagging at anaphase resulting from mitotic disturbance (*68*). MN packing acentric chromosomal fragments appear to be induced by clastogens, while MN enclosing whole chromosomes are induced by agents that affect the mitotic apparatus. The majority of DES-induced and E_2-induced MN contains whole chromosomes, which are demonstrated both with antikinetochore antibodies and with the centromere-specific DNA probe (*67*). DES may need peroxidative activation to produce metabolite(s) that induce MN, because both DES oxidation and MN induction by DES are markedly decreased by indomethacin, an inhibitor of prostaglandin H synthase activity (*66,69*). E_2 may require metabolic activation for MN induction as well, as indicated by the following: *a*) 2-OH E_2, an E_2 metabolite converted by 2-hydroxylase, binds covalently to tubulin *in vitro* with or without peroxidative activation system (*55*), but E_2 itself does not; and *b*) E_2 exhibits comparable MN induction to DES in SHE cells (*64*), which have both oxidative (*28*) and peroxidative activities (*29*). Schnitzler et al. (*67*) have shown that the mechanism of DES-induced MN is different from that of E_2-induced MN using SHE cells and ovine seminal cells. DES-induced MN can arise through chromosome nondisjunction due to spindle disruption, whereas E_2 at the concentrations used in the MN assay exerts no detectable effect on the formation of the mitotic spindle, but causes chromosome dislocation, probably due to a functional loss of the mitotic apparatus.

Genotoxicity

Although DES is not genotoxic in many assays, in certain studies DES has been found to induce UDS (*21,70–72*), sister chromatid exchanges (*73–75*), chromosome aberrations (*76,77*) and gene mutations (*33,78*). The positive studies of DNA damage by DES use either cultured mammalian cells with exogenous metabolic activation or cells with possible endogenous activation capacity for DES. Therefore, we directly compared the cell transforming activity and genotoxicity of DES in the same cells with and without exogenous metabolic activation. When SHE cells are treated in the absence of a rat liver PMS-metabolic activation system, DES fails to induce DNA damage in SHE cells at doses that induce cell transformation (*7,19,20, 21,33*). However, treatment of SHE cells with DES in the presence of an exogenous metabolic activation system enhances the frequency of morphological transformation of the cells. Furthermore, this treatment elicits UDS and gene mutations in the cells at the Na^+/K^+-ATPase locus (*33*). Thus, we have proposed two potential mechanisms for estrogen-induced cell transformation; in one the target of the estrogen is not DNA but rather microtubule disruption and the other is associated with DNA damage (*33*). Both pathways may involve active genotoxic metabolites of DES.

DNA Adduct Formation

Cellular DNA damage induced by chemicals can be examined by detection of DNA adduct formation through a covalent modification of DNA. Liehr et al. (*79*) demonstrated the presence of covalent DNA adducts in premalignant kidneys of Syrian hamsters treated chronically with DES using a sensitive ^{32}P-postlabeling assay. Because structurally diverse estrogens induced identical DNA adducts, they concluded that estrogens induce binding of the same unknown endogenous compounds to target tissue DNA. They also reported that a distinct pattern of DNA adducts was detected in the liver, kidney, uterus, and testes of Syrian hamsters following treatment with DES (*80*), and the major adducts found were similar to those produced by reaction of diethylstilbestrol-4′,4″-quinone with DNA (*39*). This suggests that DES acts as a genotoxic carcinogen via its metabolic activation to the electrophilic 4′,4″-quinone (*39*). There is another possible mechanism by which DES may cause DNA damage. Microsome-mediated redox cycling between DES or its catechol and the corresponding quinones generates superoxide radicals ($O_2^{-\bullet}$) and hydroxyl radicals ($OH^\bullet$) (*81–84*). Free radicals generated by the redox cycling of DES also oxidize 2′-deoxyguanosine to 8-hydroxy-2′-deoxyguanosine (8-OH-dG) *in vitro* (*85*) as well as *in vivo* (*86*). Small DNA adducts such as 8-OH-dG could be a causal basis for DES-induced DNA damage, which is detected as gene mutations and UDS in cultured SHE cells (*21,33*).

Chronic exposure of Syrian hamsters to E_2 for 6 months induces renal tumors (*6*), and the treatment causes covalent DNA alterations (adduct formation) in the kidney (*82*). E_2 is metabolically oxidized to catechol estrogens (2-OH E_2 and 4-OH E_2), which are postulated to be capable of

redox cycling (*84*). Free radical-mediated DNA damage might be involved in E_2 carcinogenesis because 8-OH-dG levels increase in kidney DNA of male Syrian hamsters in chronic treatment with E_2 (*87*).

To study the possible involvement of DNA damage in cell transformation induced by estrogens, we have examined whether DNA adduct formation is elicited in SHE cells treated with estrogens and their metabolites by means of the nuclease P1 enhancement version of ^{32}P-postlabeling (*30*). DNA adduct formation is detected in SHE cells treated with DES, but not in SHE cells treated with *trans, trans*-dienestrol (α-dienestrol) or β-dienestrol. Similarly, morphological transformation of SHE cells is induced by DES, but not by α- or β-dienestrol. Treatment of SHE cells with DES in the presence of exogenous metabolic activation with rat liver PMS enhances morphological transformation in a dose-dependent manner. Exposure of SHE cells to DES under the same conditions with exogenous metabolic activation induces somatic mutations and UDS. However, following this treatment, DNA adduct formation is not detected in SHE cells. It is possible that DNA adducts may be formed but not detected because of the instability of the adducts (*39*). However, this is unlikely because DNA adduct formation is not detected even when SHE cells are treated with DES for 30 min (*30*). Exposure of SHE cells to E_2 and its metabolites, 2-OH E_2 and 4-OH E_2, for 24 hr leads to covalent DNA adduct formation, corresponding to the induction of cell transformation (*30*). The results indicate that estrogens induce DNA adduct formation in cultured SHE cells, but the induction may not be the only mechanism relevant to the initiation of cell transformation. Because DES and E_2 result in DNA adduct formation and aneuploidy in SHE cells, the possibility exists that DNA adduct formation is involved in nondisjunction leading to aneuploidy. Alternatively, DNA adduct formation may only correlate with other adducts in different critical macromolecules, such as tubulin (*55,88,89*), and may not be causally involved in either morphological transformation or aneuploidy induction.

E_2 is metabolically oxidized to catechol estrogens that are also postulated to be capable of redox cycling, which would generate free radicals by redox cycling between 2- or 4-OH E_2 and their corresponding quinones (*82*). Roy et al. (*86*) showed that 8-OH-dG levels increase in kidney and liver DNAs of male Syrian hamsters by chronic treatment with DES but not with E_2. This suggests the involvement of different mechanisms in cell transformation induced by DES and E_2.

In summary, estrogens (DES and E_2) and their metabolites induce morphological transformation of SHE cells in a dose-related manner. DES and E_2 do not cause significant increases in the chromosome aberrations in SHE cells, but induce numerical chromosome changes in the near diploid range, corresponding to the transforming activity. In addition, these estrogens result in DNA adduct formation in SHE cells. It has not been clear which cytogenetic endpoints are more correlated on a causal basis with the estrogen-induced cell transformation. Moreover, other effects by estrogens, e.g., covalent binding to proteins (*55,88,89*) and generation of reactive oxygen species (*39,81,83,84,86*) could participate into inducing transformed cells. Furthermore, we cannot rule out the possibility that multiple effects of estrogens act together to cause genetic alterations leading to cell transformation. Our studies do, however, suggest that estrogens have the ability to directly transform cells by multiple mutagenic mechanisms unrelated to estrogenicity. These estrogen-induced changes, in conjunction with epigenetic changes mediated through the estrogen receptor, may contribute to hormonal carcinogenesis.

REFERENCES

1. Pike M, Spicer DV. Endogenous estrogen and progesterone as the major determinants of breast cancer risk: prospects for control by "natural" and "technological" means. In: Hormonal Carcinogenesis (Li JJ, Nandi S, Li SA, eds). New York: Springer-Verlag, 1991;209–216.
2. Brinton LA, Hoover R, Fraumeni JF Jr. Menopausal estrogen and breast cancer risk. An expanded case-control study. Br J Cancer 54:825–832 (1986).
3. Huff J, Boyd J, Barrett JC. Cellular and Molecular Mechanisms of Hormone Carcinogenesis: Environmental Influences. New York:Wiley-Liss, 1996.
4. IARC. IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans, Sex Hormones (II). Vol 21. Lyon:International Agency for Research on Cancer, 1970;139–362.
5. Kirkman H. Estrogen-induced tumors of the kidney. III: Growth characteristics in the Syrian hamster. J Natl Cancer Inst Monogr 1:1–58 (1959).
6. Herbst AL, Ulfelder H, Poskanzer DC. Adenocarcinoma of the vagina: association of maternal stilbestrol therapy with tumor appearance in young women. N Engl J Med 284:878–881 (1979).
7. Barrett JC, Hesterberg TW, Oshimura M, Tsutsui T. Role of chemically induced mutagenic events in neoplastic transformation of Syrian hamster embryo cells. In: Carcinogenesis: A Comprehensive Survey. Vol 9: Mammalian Cell Transformation Assays. New York:Raven Press, 1985;123–127.
8. Grubbs CJ, Peckham JC, McDonougt KD. Effects of ovarian hormones on the induction of 1-methyl-nitrosourea-induced mammary cancer. Carcinogenesis 4:495–497 (1983).
9. Noronha RFX, Goodal CM. The effects of estrogen on single dose dimethylnitrosamine carcinogenesis in male inbred Cr1/CDF rats. Carcinogenesis 5:1003–1007 (1984).
10. Yager JD Jr, Yager R. Oral contraceptive steroids as promoters of hepatocarcinogenesis in female Sprague-Dawley rats. Cancer Res 40:3680–3685 (1980).
11. Yager JD, Campbell HA, Longneck DS, Roebuck BD, Benoi MC. Enhancement of hepatocarcinogenesis in female rats by ethynyl estradiol and mestranol but not estradiol. Cancer Res 44:3862–3869 (1984).
12. Sheehan DM, Frederick CB, Branham S, Heath JE. Evidence for estradiol promotion of neoplastic lesions in the rat vagina after initiation with *N*-methyl-*N*-nitrosourea. Carcinogenesis 3:957–959 (1982).
13. Waldron J. Interpretation of epidemiological studies of human cancer in relation to multistep models of carcinogenesis. In: Mechanisms of Environmental Carcinogenesis. Vol II: Mechanisms of Multistep Carcinogenesis (Barrett JC, ed). Boca Raton, FL:CRC Press, 1987;21–58.
14. Moolgavkar SH, Knudson AG. Mutation and cancer: a model for human carcinogenesis. J Natl Cancer Inst 66:1037–1052 (1981).
15. Satyaswaroop PG, Zaino RJ, Mortel R. Human endometrial adenocarcinoma transplanted into nude mice: growth regulation by estradiol. Science 219:58–60 (1983).

16. Barrett JC, Huff J. Cellular and molecular mechanisms of chemically induced carcinogenesis. Renal Failure 13:211–225 (1991).
17. Li JJ, Li SA, Oberley T. Estrogen carcinogenicity: hormonal morphologic and chemical interactions. In: Chemical Carcinogens. Activation Mechanisms, Structural and Electronic Factors, and Reactivity (Politzer P, Martin FJ Jr, eds). New York:Elsevier, 1988;312–321.
18. Newbold RR, Bullock BC, McLachlan JA. Uterine adenocarcinoma in mice following developmental treatment with estrogens: a model for hormonal carcinogenesis. Cancer Res 50:7677–7681 (1990).
19. Barrett JC, Wong A, McLachlan JA. Diethylstilbestrol induces neoplastic transformation without measurable gene mutation at two loci. Science 212:1402–1404 (1981).
20. Tsutsui T, Maizumi H, McLachlan JA, Barrett JC. Aneuploidy induction and cell transformation by diethylstilbestrol: a possible chromosomal mechanism in carcinogenesis. Cancer Res 43:3814–3821 (1983).
21. Tsutsui T, Degen GH, Schiffmann D, Wong A, Maizumi H, McLachlan JA, Barrett JC. Dependence on exogenous metabolic activation for induction of unscheduled DNA synthesis in Syrian hamster embryo cells by diethylstilbestrol and related compounds. Cancer Res 44:184–189 (1984).
22. Barrett JC, Oshimura M, Tsutsui T, Tanaka N. Role of aneuploidy in early and late stages of neoplastic progression of Syrian hamster embryo cells in culture. In: Aneuploidy: Etiology and Mechanisms (Dellarco VL, Voytek PE, Hollaender A, eds). New York:Plenum Press, 1985;523–538.
23. Tsutsui T, Suzuki N, Fukuda S, Sato M, Maizumi H, McLachlan JA, Barrett JC. 17β-Estradiol-induced cell transformation and aneuploidy of Syrian hamster embryo cells in culture. Carcinogenesis 8:1715–1719 (1987).
24. Barrett JC, Tsutsui T. Mechanisms of estrogen-associated carcinogenesis. In: Cellular and Molecular Mechanisms of Hormonal Carcinogenesis: Environmental Influences (Huff J, Boyd J, Barrett JC, eds). New York:Wiley-Liss, 1996;105–112.
25. McLachlan JA, Wong A, Degen GH, Barrett JC. Morphological and neoplastic transformation of Syrian hamster embryo cells by diethylstilbestrol and its analogs. Cancer Res 42:3040–3045 (1982).
26. Ernst H, Riebe M, Mohr U. Undifferentiated sarcomas induced in Syrian hamsters by subcutaneous injection of diethylstilbestrol. Cancer Lett 31:181–186 (1986).
27. Korach KS, McLachlan JA. The role of the estrogen receptor in diethylstilbestrol toxicity. Arch Toxicol 58(Suppl 8):33–42 (1985).
28. Pienta RJ. Transformation of Syrian hamster embryo cells by diverse chemicals and correlation with their reported carcinogenic and mutagenic activities. In: Chemical Mutagens, Principles and Methods for Their Detection, Vol 6 (deSerres FJ, Hollaender A, eds). New York/London:Plenum Press, 1980;175–202.
29. Degen GH, Wong A, Eling TE, Barrett JC, McLachlan JA. Involvement of prostaglandin synthetase in the peroxidative metabolism of diethylstilbestrol in Syrian hamster embryo fibroblast cell cultures. Cancer Res 43:992–996 (1983).
30. Hayashi N, Hasegawa K, Barrett JC, Tsutsui T. Estrogen-induced cell transformation and DNA adduct formation in cultured Syrian hamster embryo cells. Mol Carcinog 16:149–156 (1996).
31. Metzler M, McLachlan JA. Peroxidase-mediated oxidation, a possible pathway for metabolic activation of diethylstilbestrol. Biochem Biophys Res Commun 85:874–884 (1978).
32. Maydl R, Newbold RR, Metzler M, McLachlan JA. Organ cultures of the fetal mouse genital tract metabolize diethylstilbestrol (DES). Proc Am Assoc Cancer Res 22:104 (1981).
33. Tsutsui T, Suzuki N, Maizumi H, McLachlan JA, Barrett JC. Alteration in diethylstilbestrol-induced mutagenicity and cell transformation by exogenous metabolic activation. Carcinogenesis 7:1415–1418 (1986).
34. Fitzgerald DJ, Piccoli C, Yamasaki H. Detection of non-genotoxic carcinogens in the BALB/c 3T3 cell transformation/mutation assay system. Mutagenesis 4:286–291 (1989).
35. Rinehart CA, Xu L-H, Le LV, Kaufman DG. Diethylstilbestrol-induced immortalization of human endometrial cells: alterations in *p53* and estrogen receptor. Mol Carcinog 15:115–123 (1996).
36. Fishman J, Hellman L, Zumoff B, Cassouto J. Pathway and stereochemistry of the formation of estriols in man. Biochemistry 5:1789–1794 (1996).
37. Fishman J. Aromatic hydroxylation of estrogens. Annu Rev Physiol 45:61–72 (1983).
38. Li SA, Klicka JK, Li JJ. Estrogen 2- and 4-hydroxylase activity, catechol estrogen formation, and implications for estrogen carcinogenesis in the hamster kidney. Cancer Res 45:181–185 (1985).
39. Liehr JG. Genotoxic effects of estrogens. Mutat Res 238:269–276 (1990).
40. Telang NT, Suto A, Wong GY, Osborne MP, Bradlow HL. Induction by estrogen metabolite 16α-hydroxyestrone of genotoxic damage and aberrant proliferation in mouse mammary epithelial cells. J Natl Cancer Inst 84:634–638 (1992).
41. Yu SC, Fishman J. Interaction of histones with estrogens. Covalent adduct formation with 16α-hydroxyestrone. Biochemistry 24:8017–8021 (1985).
42. Swaneck GE, Fishman J. Covalent binding of the endogenous estrogen 16α-hydroxyestron to estradiol receptor in human breast cancer cells: characterization and intranuclear localization. Proc Natl Acad Sci USA 85:7831–7835 (1988).
43. Schneider J, Kinne D, Fracchia A, Pierce V, Anderson KE, Bradlow HL, Fishman J. Abnormal oxidative metabolism of estradiol in women with breast cancer. Proc Natl Acad Sci USA 79:3047–3051 (1982).
44. Telang NT, Bradlow HL, Kurihara H, Osborne NP. *In vitro* biotransformation of estradiol by explant cultures of murine mammary tissues. Breast Cancer Res Treat 13:173–181 (1989).
45. Liehr JG, Purdy RH, Baran JS, Nutting EF, Colton F, Randerath E, Randerath K. Correlation of aromatic hydroxylation of 11β-substituted estrogens with morphological transformation *in vitro* but not with *in vivo* tumor induction by these hormones. Cancer Res 47:2583–2588 (1987).
46. Tsutsui T, Taguchi S, Tanaka Y, Barrett JC. 17β-estradiol, diethylstilbestrol, tamoxifen, toremifene and ICI 164,384 induce morphological transformation and aneuploidy in cultured Syrian hamster embryo cells. Intl J Cancer 70:188–193 (1997).
47. Wakeling AE, Bowel J. Biology and model of action of pure antiestrogens. J Steroid Biochem 30:141–147 (1988).
48. Ozawa N, Oshimura M, McLachlan JA, Barrett JC. Nonrandom karyotypic changes in immortal and tumorigenic Syrian hamster cells induced by diethylstilbestrol. Cancer Genet Cytogenet 38:271–282 (1989).
49. Tucker RW, Barrett JC. Decreased numbers of spindle and cytoplasmic microtubules in hamster embryo cells treated with a carcinogen, diethylstilbestrol. Cancer Res 46:2088–2095 (1986).
50. Sakakibara Y, Saito I, Ichinoseki K, Oda T, Kaneko M, Saito H, Kodama M, Sato Y. Effects of diethylstilbestrol and its methyl ethers on aneuploidy induction and microtubule distribution in Chinese hamster V79 cells. Mutat Res 263:269–276 (1991).
51. Aizu-Yokota E, Ichinoseki K, Sato Y. Microtubule disruption induced by estradiol in estrogen receptor-positive and -negative human breast cancer cell lines. Carcinogenesis 15:1875–1879 (1994).
52. Sato Y, Sakakibara Y, Oda T, Aizu-Yokota E, Ichinoseki K. Effect of estradiol and ethynylestradiol on microtubule distribution in Chinese hamster V79 cells. Chem Pharm Bull 40:182–184 (1992).
53. Aizu-Yokota E, Susaki A, Sato Y. Natural estrogens induce modulation of microtubules in Chinese hamster V79 cells in culture. Cancer Res 55:1863–1868 (1995).

54. Sato Y, Murai T, Tsumuraya M, Saito H, Kodama M. Disruptive effect of diethylstilbestrol on microtubules. Jpn J Cancer Res (Gann) 75:1046–1048 (1984).
55. Epe B, Heglar J, Metzler M. Site-specific covalent binding of stilbene-type and steroidal estrogens to tubulin following metabolic activation *in vitro*. Carcinogenesis 8:1271–1275 (1987).
56. Bradley MO, Bhuyan B, Francis MC, Langenbach R, Peterson A, Huberman E. Mutagenesis by chemical agent in V79 Chinese hamster cells: a review and analysis of the literature. Mutat Res 87:81–142 (1981).
57. Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD, eds. Molecular Biology of the Cell. New York/London:Garland Publishing, 1993.
58. Hsie AW, Puck TT. Morphological transformation of Chinese hamster cells by dibutyryl adenosine cyclic 3':5'-monophosphate and testosterone. Proc Natl Acad Sci USA 68:358–361 (1971).
59. Browne CL, Lockwood AH, Su JL, Beavo JA, Sterner AL. Immunofluorescent localization of cyclic nucleotide-dependent protein kinases on the mitotic apparatus of cultured cells. J Cell Biol 87:336–345 (1980).
60. Edwards K, Laughton C, Neidle S. A molecular modeling study of the interactions between the antiestrogen drug tamoxifen and several derivatives, and the calcium-binding protein calmodulin. J Med Chem 35:2753–2761 (1992).
61. Sargent LM, Dragen YP, Bahnub N, Wiley JE, Sattler CA, Schroeder P, Sattler GL, Jordan VC, Pitot HC. Tamoxifen induces hepatic aneuploidy and mitotic spindle disruption after a single *in vivo* administration to female Sprague-Dawley rats. Cancer Res 54:3357–3360 (1994).
62. Geiser JR, Sundberg HA, Chang BH, Muller EGD, Davis TN. The essential mitotic target of calmodulin is the 110-kilodalton component of the spindle pole body in *Saccharomyces cerevisiae*. Mol Cell Biol 13:7913–7924 (1993).
63. Wheeler WJ, Hsu TC, Tousson A, Brinkley BR. Mitotic inhibition and chromosome displacement induced by estradiol in Chinese hamster cells. Cell Motil Cytoskel 7:235–247 (1987).
64. Schmuck G, Lieb G, Wild D, Schiffmann D, Henschler D. Characterization of an *in vitro* micronucleus assay with Syrian hamster embryo fibroblasts. Mutat Res 203:397–404 (1988).
65. Schiffmann D, DeBoni U. Dislocation of chromatin elements in prophase induced by diethylstilbestrol: a novel mechanism by which micronuclei can arise. Mutat Res 246:113–122 (1991).
66. Foth J, Schnitzler R, Jager M, Koob M, Metzler M, Degen GH. Characterization of sheep seminal vesicle cells—a new tool for studying genotoxic effects *in vitro*. Toxicol in Vitro 6:219–225 (1992).
67. Schnitzler R, Foth J, Degen GH, Metzler M. Induction of micronuclei by stilbene-type and steroidal estrogens in Syrian hamster embryo and ovine seminal vesicle cells *in vitro*. Mutat Res 311:85–93 (1994).
68. Bahari IB, Noor FM, Daud MM. Micronucleated erythrocytes as an assay to assess actions by physical and chemical genotoxic agents in *Clarias gariepinus*. Mutat Res 313:1–5 (1994).
69. Foth J, Degen GH. Prostaglandin H synthase dependent metabolism of diethylstilbestrol in ram seminal vesicle cell cultures. Arch Toxicol 65:344–347 (1991).
70. Martin CN, McDermid AC, Garner RC. Testing of known carcinogens and noncarcinogens for their ability to induce unscheduled DNA synthesis in HeLa cells. Cancer Res 38:2621–2627 (1978)
71. Martin CN, McDermid AC. Testing of 42 coded compounds for their ability to induce unscheduled DNA repair synthesis in the HeLa cells. In: Evaluation of Short-term Tests for Carcinogens (deSerres FJ, Ashby J, eds). New York: Elsevier/North Holland, 1982;533–537.
72. Robinson DE, Mitchell AD. Unscheduled DNA synthesis response to human fibroblast WI-38 cells to 20 coded chemicals. In: Evaluation of Short-term Tests for Carcinogens (deSerres FJ, Ashby J, eds). New York:Elsevier/North Holland, 1982;517–527.
73. Rüdiger HW, Haenish F, Metzler M, Oesch F, Glatt HR. Metabolites of diethylstilbestrol induce sister chromatid exchange in human cultured fibroblasts. Nature 281:392–394 (1979).
74. Hill A, Wolff S. Increased induction of sister chromatid exchange by diethylstilbestrol in lymphocytes from pregnant and premenopausal women. Cancer Res 42:893–896 (1982).
75. Buenaventura SK, Jacobson-Kram D, Dearfield KL, Williams JR. Induction of sister chromatid exchange by diethylstilbestrol in metabolically competent hepatoma cell lines but not in fibroblasts. Cancer Res 44:3851–3855 (1984).
76. Bishun N, Forster S, Valera N, Williams DS. The clastogenic effects of diethylstilbestrol on ascitic tumor cell *in vivo*. Microbios Lett 13:27–31 (1980).
77. Ivett JL, Tice RR. Diethylstilbestrol-diphosphate induces chromosomal aberrations but not sister chromatid exchanges in murine bone marrow cells *in vivo*. Environ Mutagen 3:445–452 (1981).
78. Clive D, Johnson KO, Spector JFS, Batson AG, Brown MMM. Validation and characterization of the L5173Y/TK$^{+/-}$ mouse lymphoma mutagen assay system. Mutat Res 59:61–108 (1979).
79. Liehr JG, Avitts TA, Randerath E, Randerath K. Estrogen-induced endogenous DNA adduction: possible mechanism of hormonal cancer. Proc Natl Acad Sci USA 83:5301–5305 (1986).
80. Gladek A, Liehr JG. Mechanism of genotoxicity of diethylstilbestrol *in vivo*. J Biol Chem 264:16847–16852 (1989).
81. Epe B, Schiffmann D, Metzler M. Possible role of oxygen radicals in cell transformation by diethylstilbestrol and related compounds. Carcinogenesis 7:1329–1334 (1986).
82. Lieher JG, Ulubelen AA, Strobel HW. Cytochrome P-450-mediated redox cycling of estrogens. J Biol Chem 261:16865–16870 (1986).
83. Roy D, Liehr JG. Temporary decrease in renal quinone reductase activity induced by chronic administration of estradiol to male Syrian hamsters: increased superoxide formation by redox cycling of estrogen. J Biol Chem 263:3646–3651 (1988).
84. Liehr JG, Roy D. Free radical generation by redox cycling of estrogens. Free Radic Biol Med 8:415–423 (1990).
85. Roseier JA, Van Peteghem CH. Peroxidative *in vitro* metabolism of diethylstilbestrol induces formation of 8-hydroxy-2′-deoxyguanosine. Carcinogenesis 10:405–406 (1989).
86. Roy D, Froyd RA, Liehr JG. Elevated 8-hydroxydeoxy- guanosine levels in DNA of diethylstilbestrol-treated Syrian hamsters: covalent DNA damage by free radicals generated by redox cycling of diethylstilbestrol. Cancer Res 51:3882–3885 (1991).
87. Han X, Liehr JG. 8-Hydroxylation of guanine bases in kidney and liver DNA of hamsters treated with estradiol: role of free radicals in estrogen-induced carcinogenesis. Cancer Res 54:5515–5517 (1994).
88. Haaf H, Metzler M. Covalent binding of diethylstilbestrol to microsomal protein *in vitro* correlates with the organotropism of its carcinogenicity. Carcinogenesis 6:659–660 (1985).
89. Epe B, Harttig U, Stopper H, Metzler M. Covalent binding of reactive estrogen metabolites to microtubular protein as a possible mechanism of aneuploidy induction and neoplastic cell transformation. Environ Health Perspect 88:123–127 (1990).